Plasma cathepsin d levels: a novel tool to predict pediatric hepatic inflammation

Plasma Cathepsin D Levels: A Novel Tool to Predict Pediatric Hepatic Inflammation

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OBJECTIVES: **Nonalcoholic steatohepatitis (NASH) is the most severe form of a hepatic condition known as nonalcoholic fatty liver disease (NAFLD). NASH is histologically characterized by hepatic fat accumulation, inflammation, and ballooning, and eventually coupled with fibrosis that, in turn, may progress to end-stage liver disease even in young individuals. Hence, there is a critical need for specific noninvasive markers to predict hepatic inflammation at an early age. We investigated whether plasma levels of cathepsin D (CatD), a lysosomal protease, correlated with the severity of liver inflammation in pediatric NAFLD.**

METHODS: **Liver biopsies from children (*n*=96) with NAFLD were histologically evaluated according to the criteria of Kleiner (NAFLD activity score) and the Brunt's criteria. At the time of liver biopsy, blood was taken and levels of CatD, alanine aminotransferase (ALT), and cytokeratin-18 (CK-18) were measured in plasma.**

RESULTS: **Plasma CatD levels were significantly lower in subjects with liver inflammation compared with steatotic subjects. Furthermore, we found that CatD levels were gradually reduced and corresponded with increasing severity of liver inflammation, steatosis, hepatocellular ballooning, and NAFLD activity score. CatD levels correlated with pediatric NAFLD disease progression better than ALT and CK-18. In particular, CatD showed a high diagnostic accuracy (area under receiver operating characteristic curve (ROC-AUC): 0.94) for the differentiation between steatosis and hepatic inflammation, and reached almost the maximum accuracy (ROC-AUC: 0.998) upon the addition of CK-18.**

CONCLUSIONS: **Plasma CatD holds a high diagnostic value to distinguish pediatric patients with hepatic inflammation from children with steatosis.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

The current obesity epidemic in children is paralleled by an increasing prevalence of nonalcoholic fatty liver disease (NAFLD), a condition characterized by early hepatic steatosis and by a more severe form known as nonalcoholic steatohepatitis (NASH). Key histologic components of NASH are steatosis, inflammation, and hepatocellular ballooning (1). Children with biopsy-proven NAFLD were followed up for 20 years and demonstrated a shorter long-term survival. Some children evolved toward advanced fibrosis, others to cirrhosis, and a 20-year-old girl needed a liver transplantation owing to end-stage liver disease, hereby showing the progressive nature of NAFLD even in young individuals (2). Therefore, as NASH progresses to these more severe chronic liver conditions, its early detection is crucial to design adequate

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patient management and to reduce end-stage liver disease and overall mortality. Unfortunately, NASH cannot be identified without pursuing a liver biopsy, the reference standard for accurate staging and grading of NAFLD. Owing to the invasiveness of this method, the high costs, the level of discomfort, sampling error, and its risk for complications such as hemorrhage and bile peritonitis (3), liver biopsies, especially at a young age, should be replaced with noninvasive markers to detect NASH.

In addition to plasma alanine aminotransferase (ALT), a number of functionally diverse biomolecules could be used as potential markers for pediatric NASH, including several proteins that change their expression and stability during the development of steatosis, inflammation, and ballooning (4). An extensively studied molecule is the caspase-cleaved cytokeratin-18 (CK-18) that, in addition to its ability to detect hepatocellular apoptosis, is also able to distinguish NASH from simple steatosis in both adults and children (5,6).

Previously, we demonstrated a clear and direct association between hepatic inflammation and lysosomal cholesterol accumulation inside Kupffer cells (KCs) of low-density lipoprotein receptor knockout (*Ldlr*−/−) mice fed a high-fat, high-cholesterol diet (7–9). In line with our observation in mice, cholesterol-containing KCs were also demonstrated recently in livers of NASH patients (10). Further studies have shown that lysosomal cholesterol accumulation induces disturbances in lysosomal (enzyme) trafficking (11,12). In particular, cathepsins, the main class of lysosomal proteases, have been described to have an early role in inflammation (13). In line, recent evidence showed a disruption of hepatic cathepsin expression in NAFLD patients (14).

We hypothesized that cathepsin D (CatD) levels in plasma may be altered in subjects with NASH compared with subjects without NASH (i.e., steatosis). Therefore, the aim of this study was to assess plasma levels of CatD in children with different stages of NAFLD and to correlate these to histological criteria used for the diagnosis of NASH. In addition, receiver operating characteristic (ROC) curves were plotted to test the diagnostic accuracy of CatD.

METHODS

Sample collection for this study was performed at Hepato-Metabolic Department of Bambino Gesù Children's Hospital during the period September 2012–December 2013. The study design conformed to the ethical guidelines of the Declaration of Helsinki (1975) and was performed according to the recommendations of the Ethics Committee of the Hospital.

We included consecutive children aged 3 to 12 years with an ultrasonographic diagnosis of NAFLD and persistently (≥6 months) elevated serum aminotransferases. The decision to biopsy NAFLD children was based on our previously published data, demonstrating a high prevalence of necroinflammatory changes and fibrosis in children with ultrasonographic steatosis and persistently elevated liver enzymes (15). Blood samples were obtained just before the ultrasound-guided liver biopsy. All blood samples were originally processed to yield plasma and stored at −80 °C. Exclusion criteria were hepatic virus infections (hepatitis A, B, C, cytomegalovirus, and Epstein–Barr virus), type 1 diabetes mellitus, excessive alcohol consumption (≥140 g/week), history of parenteral nutrition, and use of drugs known to induce steatosis (e.g., valproate, amiodarone, or prednisone) or to affect body weight and carbohydrate metabolism. Autoimmune liver disease, metabolic liver disease, Wilson's disease, celiac disease, type 2 diabetes mellitus, and α-1-antitrypsin deficiency were ruled out using standard clinical, laboratory, and histological criteria (16,17).

Liver histology

The clinical indication for liver biopsy was either to assess and better define the presence of NASH and the degree of fibrosis or to exclude other likely liver diseases (16). A Sonoline Omnia ultrasound machine (Siemens, Munich, Germany) equipped with a 5-MHz probe (5.0C50, Siemens) and a biopsy adaptor was used.

The liver specimens of ≥15 mm length including at least 5–6 complete portal tracts were considered adequate for the purpose of the study. Biopsies were routinely processed (i.e., formalin-fixed and paraffin-embedded) and sections of liver tissue were stained with hematoxylin–eosin, Van Gieson, periodic acid–Schiff diastase, and Prussian blue stain. Biopsies were evaluated by a single expert hepatopathologist who was unaware of the patient's clinical and laboratory data. To determine the intraobserver agreement, the pathologist scored the liver biopsies blindly twice, and the weighted κ-coefficients for different histological features were calculated.

The main histological features of NAFLD/NASH, including steatosis, inflammation, hepatocellular ballooning, and fibrosis, were scored using the NAFLD Clinical Research Network criteria (18). Briefly, steatosis was graded on a 4-point scale: grade 0=steatosis involving <5% of hepatocytes; grade 1=steatosis involving up to 33% of hepatocytes; grade 2=steatosis involving 34–65% of hepatocytes; and grade 3=steatosis involving ≥66% of hepatocytes. Lobular inflammation was graded on a 4-point scale: grade 0=no foci; grade 1=<2 foci per 200× field; grade 2=2–4 foci per 200× field; and grade 3=>4 foci per 200× field. Hepatocellular ballooning was graded from 0 to 2: 0=none; 1=few balloon cells; and 2 =many/prominent balloon cells. The stage of fibrosis was quantified using a 5-point scale: stage 0=no fibrosis; stage 1=perisinusoidal or periportal (1a=mild, zone 3, perisinusoidal; 1b=moderate, zone 3, perisinusoidal; 1c=portal/periportal); stage 2=perisinusoidal and portal/periportal; stage 3=bringing; and stage 4=cirrhosis. Features of steatosis, lobular inflammation, and hepatocellular ballooning were combined to obtain the NAFLD activity score (NAS score). The NAS score ranged from 1 to 7. As recently recommended by NASH Clinical Research Network (19), a microscopic diagnosis based on overall injury pattern (steatosis, hepatocellular ballooning, inflammation), as well as the presence of additional lesions (e.g., zonality of lesions, portal inflammation, and fibrosis), has been assigned to each case. Accordingly, biopsies were subdivided into steatosis, borderline NASH, and definite NASH (19).

Laboratory assessment

At the time of the liver biopsy, blood was taken for further analysis. In all patients enrolled in this study, aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase, triglycerides, total

cholesterol, albumin levels, glucose tolerance, and prothrombin time (international normalized ratio) were evaluated using standard laboratory methods. Insulin resistance was calculated according to the Homeostatic Model Assessment-Insulin Resistance derived from basal values of glucose and insulin, as previously described (20).

Human CatD enzyme-linked immunosorbent assay

Plasma samples were diluted and CatD levels were determined by the CatD enzyme-linked immunosorbent assay according to the manufacturer's protocol (Uscn Life Science, Wuhan, China). The absorbance was measured on a Benchmark 550 microplate reader (Bio-Rad, Hercules, CA). The detection limit ranges approximately from 46.88 to 3,000 pg/ml. Coefficients of variation for intra- and inter-assays are <10 and <12%, respectively. The CatD measurements were performed blinded to the histology findings of the study participants.

CK-18 level measurements

Part of plasma samples were used for quantitative determination of CK-18 levels by the M30-Apoptosense ELISA kit (PEVIVA) purchased from Li Starfish (Milan, Italy). All assays were performed in duplicate, and the absorbance was determined using a microplate reader (Molecular Bio-Rad, Milan, Italy).

Statistical analysis

The data were analyzed by performing one-way analysis of variance and *post hoc* Tukey's test. The data were expressed as mean±s.e.m. and considered significant at $P<0.05$. To evaluate the performance of plasma CatD, the sensitivity, specificity, predictive values, and area under the curve (AUC) were calculated using ROC curve analysis for the following differentiations: steatosis vs. NASH, borderline NASH vs. NASH, steatosis + borderline NASH vs. NASH and steatosis vs. borderline NASH + NASH. A statistical significance between two AUCs was evaluated by computing the $Z-\text{score} = (\text{AUC}_1 - \text{AUC}_2)/\sqrt{\left(\text{SE}^2_{AUC1} - \text{SE}^2_{AUC2}\right)}$, followed by calculating two-tailed P values. $P<0.05$ was considered significant. The analysis and the graphs were performed using GraphPad Prism 5 (version 5.03, GraphPad Software Inc., San Diego, CA).

To assess the diagnostic accuracy of the combination of CK-18 with CatD, a logistic regression was fitted to steatosis, borderline NASH, and NASH patients for both CK-18 and CatD. Statistical ROC analyses and AUC presented for the combination of CK-18 with CatD were performed using the freely available program R (21) and the publicly available libraries "gnlm" (22) and "DiagnosisMed."

RESULTS

General characteristics of the pediatric population

A total of 96 children (mean age: 8.9 years, ranging from 3.3 to 12.1 years) with NAFLD were enrolled in the study. Of these 96 children, 56 were boys and 40 were girls with a mean age of 9.3 and 8.4 years, respectively. NASH was diagnosed in 27% (26 subjects) of the study population, whereas 51 subjects (53%) were diagnosed with borderline NASH and 19 subjects (20%) with steatosis (**Table 1**). All children were obese (body mass index >95th percentile), and no difference was observed in body mass index percentile and waist circumference percentile between children with steatosis, borderline NASH, or definite NASH (**Table 2**). No differences were found in insulin resistance, glucose tolerance, the lipid profile, and coagulation profile (as indicated by the homeostatic model assessment-insulin resistance, the percentage of subjects with impaired glucose tolerance, the levels of cholesterol, triglycerides, albumin and the international normalized ratio). Liver-specific parameters including ALT, AST, and γ-glutamyl transpeptidase were similar between all the groups. In response to NAFLD severity, a gradual significant decrease was detected for plasma CatD, a lysosomal protease (steatosis vs. borderline NASH: $P=0.0017$; steatosis vs. NASH: $P<0.0001$; borderline NASH vs. NASH: $P<0.0001$). In contrast, CK-18 increased in response to NAFLD severity and could differentiate steatosis from borderline NASH ($P=0.0001$) and NASH ($P=0.0003$). No difference in CK-18 levels was observed between borderline NASH and definite NASH ($P=0.95$).

Table 1. Histological features of the pediatric subjects

	Steatosis (n=19)	Borderline NASH (n=51)	NASH (n=26)
NAS	2.2±0.3	3.9±0.2	5.7±0.1
Steatosis (%)			
5–33%	15 (78.9)	10 (19.6)	2 (7.7)
34–65%	3 (15.8)	5 (9.8)	5 (19.2)
≥66%	1 (5.3)	36 (70.6)	19 (73.1)
Inflammation (%)			
Grade 0	5 (26.3)	1 (2.0)	0
Grade 1	13 (68.4)	33 (64.7)	12 (46.2)
Grade 2	1 (5.3)	17 (33.3)	14 (53.8)
Ballooning (%)			
None	17 (89.5)	22 (43.1)	3 (11.5)
Few	1 (5.3)	24 (47.1)	15 (57.7)
Many	1 (5.3)	5 (9.8)	8 (30.8)
Fibrosis (%)			
Stage 0	10 (52.6)	19 (37.3)	7 (26.9)
Stage 1	8 (42.1)	26 (51.0)	16 (61.5)
Stage 2	1 (5.3)	5 (9.8)	0
Stage 3	0	1 (2.0)	3 (11.5)

NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis.
NAS is represented as mean±s.e.m.

Significantly reduced levels of plasma CatD correlate with pediatric NAFLD disease severity

First, we investigated whether plasma CatD levels correlated with histological features of NAFLD. We found that plasma levels of CatD decreased significantly in parallel with the increasing degree

Table 2. Clinical characteristics of the pediatric population

				P value between groups		
	Steatosis (1) (*n*=19)	Borderline NASH (2) (*n*=51)	NASH (3) (*n*=26)	1 vs. 2	1 vs. 3	2 vs. 3
Male (%)	12 (63.2)	29 (56.9)	15 (57.7)	0.886	0.930	0.997
Age (years)	10.0±0.5	8.4±0.3	9.2±0.4	**0.016**	0.378	0.314
Weight (kg)	52.0±2.6	45.2±2.2	44.3±2.0	0.155	0.149	0.958
Length (cm)	139.9±2.8	132.4±2.4	135.0±2.7	0.158	0.531	0.753
BMI percentile	96.0±0.8	96.0±0.7	96.1±0.8	1.000	0.995	0.996
WC percentile	83.6±2.4	77.9±1.4	82.9±1.7	0.079	0.973	0.084
Obese (%)	19 (100)	47 (92.2)	24 (92.3)	0.458	0.551	1.000
Cholesterol (mg/dl)	167.3±8.6	163.3±5.1	153.3±7.6	0.915	0.427	0.506
Triglycerides (mg/dl)	94.0±8.4	111.1±8.2	125.9±21.9	0.664	0.328	0.685
ALT (U/l)	61.6±7.4	83.7±5.6	91.0±15.3	0.258	0.152	0.830
AST (U/l)	46.7±3.5	55.3±2.6	57.4±7.2	0.387	0.311	0.929
GGT (U/l)	26.0±2.5	29.1±2.7	30.0±4.4	0.814	0.769	0.981
Cathepsin D (pg/ml)	32,658±3,640	21,428±1,753	8,095±784	**0.002**	**<0.0001**	**<0.0001**
CK-18 (U/l)	261.6±24.5	350.6±11.2	356.4±6.6	**0.0001**	**0.0003**	0.950
Albumin (g/dl)	4.5±0.1	4.5±0.1	4.6±0.1	0.866	0.668	0.869
INR	1.1±0.05	1.2±0.03	1.1±0.04	0.838	0.997	0.853
IGT (%)	0 (0)	9 (17.6)	4 (15.4)	0.137	0.297	0.959
HOMA-IR	2.7±0.4	2.5±0.3	2.4±0.3	0.973	0.922	0.970

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CK-18, cytokeratin-18; GGT, γ-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment of insulin resistance; IGT, impaired glucose tolerance; INR, international normalized ratio; NASH, nonalcoholic steatohepatitis; WC, waist circumference.

Data are represented as mean±s.e.m. Bold values signify $P<0.05$. Data were statistically analyzed using one-way analysis of variance (ANOVA) tests using the SPSS software, version 22.0 (SPSS, Chicago, IL).

of NAFLD and the NAS score (**Figure 1**). Furthermore, in line with the increased presence of hepatocellular ballooning, a key histological feature of NASH, plasma CatD declined. By examining the individual stages of NAFLD, we demonstrate that, unlike late-stage fibrosis, CatD was reduced upon increasing degrees of steatosis and inflammation. Thus, plasma CatD was significantly lowered in children with NASH compared with children with either steatosis or borderline NASH and was associated with early stages of NAFLD (i.e., steatosis and inflammation).

Plasma ALT does not correlate with the histological pattern of NAFLD in children

Currently, plasma ALT is primarily used in the clinic as a noninvasive marker to detect NASH. To make a comparison between CatD and ALT, plasma ALT levels were also measured. Opposite to CatD, a trend toward increasing ALT levels in parallel with increasing NAFLD severity was found (**Figure 2**). However, this difference was not significant, neither for Brunt's nor Kleiner's criteria. Unlike CatD, ALT levels remained similar upon severe degrees of hepatocellular ballooning and steatosis. ALT levels increased with increasing severity of inflammation, but did not reach a statistically significant level. During fibrosis, a trend toward an increase in ALT levels was found between patients who displayed no fibrosis and those who demonstrate mild or severe fibrosis. Altogether, ALT is not able to differentiate between the different stages of pediatric NAFLD and is more associated with late-stage fibrosis.

CK-18 can differentiate between steatosis and NASH and correlates with some histological features of pediatric NAFLD

As previous research has shown that plasma levels of CK-18 are accurate in predicting NASH vs. no NASH in adults (5) and children (6), we point to confirm these data in our cohort. In this study, CK-18 was increased in pediatric subjects with borderline NASH and definite NASH compared with steatosis. However, CK-18 levels remained identical between children with borderline and definite NASH (**Figure 3**). CK-18 was significantly increased in subjects with a high NAS score (3–4 and 5–7) compared with children with an NAS score of 1–2. To determine whether there is an association between CK-18 and severity of NAFLD, we categorized CK-18 according to the histological stages of hepatocyte ballooning, steatosis, inflammation, and fibrosis. In this cohort, CK-18 was significantly increased upon MODERATE hepatocyte ballooning and increased along with the severity of steatosis and inflammation. No difference in CK-18 levels between the different stages of fibrosis was observed. Altogether, we confirm that

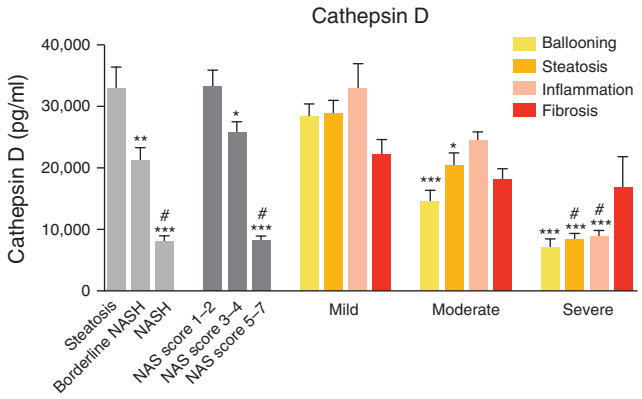

Figure 1. Cathepsin D in plasma of pediatric nonalcoholic fatty liver disease (NAFLD) subjects. Cathepsin D levels were analyzed in children with NAFLD divided by biopsy-proven steatosis, borderline nonalcoholic steatohepatitis (NASH), and NASH (light-grey bars) or divided upon the NAS score (dark-grey bars). The colored bars show plasma cathepsin D for the individual stages of NAFLD. Classification *Mild* indicates a stage 0 for hepatocellular ballooning, stage 1 for steatosis, and stage 0 for inflammation and fibrosis. Classification *Moderate* indicates stage 1 for hepatocellular ballooning, stage 2 for steatosis, and stage 1 for inflammation and fibrosis. Classification *Severe* indicates stage 2 for hepatocellular ballooning, stage 3 for steatosis, stage 2 for inflammation, and stage 2+3 for fibrosis. Asterisks denote significance compared with the corresponding first (left) bar for each classification. Each classification is indicated by the light-grey, the dark-grey, and the colored bars. **P*<0.05, ***P*<0.01, and ****P*<0.001. The hashtag (#) notation represents significance compared with the corresponding second (middle bar) for each classification. #*P*<0.001. NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis.

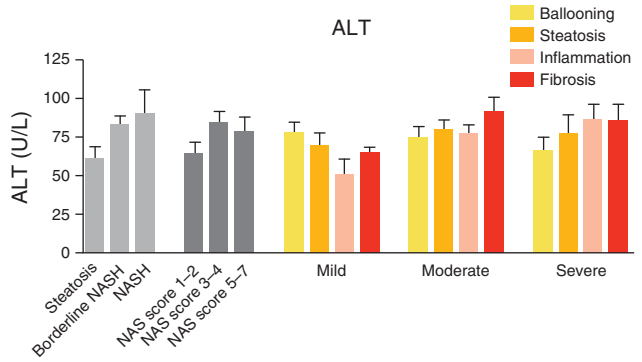

Figure 2. Plasma alanine aminotransferase (ALT) levels in children with biopsy-proven nonalcoholic fatty liver disease (NAFLD). ALT levels in children with NAFLD divided by biopsy-proven steatosis, borderline nonalcoholic steatohepatitis (NASH), and NASH (light-grey bars) or divided upon the NAS score (dark-grey bars). The colored bars show plasma ALT for the individual stages of NAFLD. NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis.

CK-18 is able to distinguish NASH from steatosis in children with NAFLD.

Plasma CatD holds a better predictive value for the differentiation between steatosis and NASH in children than CK-18 and ALT

We next investigated the clinical potential of plasma CatD for diagnosing pediatric NASH and hereby made use of plotted ROC

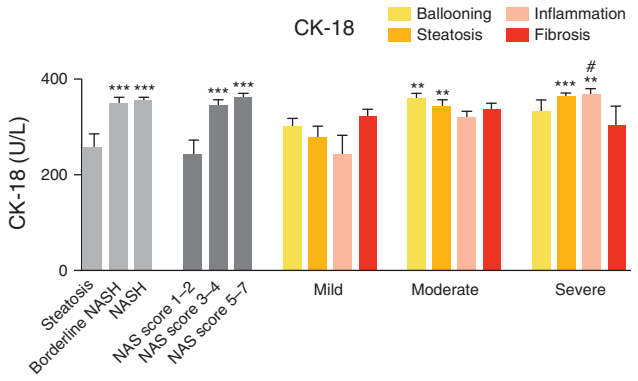

Figure 3. Cytokeratin-18 (CK-18) levels in plasma of children with nonalcoholic fatty liver disease (NAFLD). CK-18 measurements were plotted against the several stages of NAFLD. Asterisks denote significance compared with the corresponding first (left) bar for each classification. Each classification is indicated by the light-grey, the dark-grey, and the colored bars. **P*<0.05, ***P*<0.01, and ****P*<0.001. The hashtag (#) notation represents significance compared with the corresponding second (middle bar) for each classification. #*P*<0.001. NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis.

curves. For the comparison between steatosis and NASH, the ROC curve of CatD demonstrated a significant higher AUC of 0.94 as compared with the AUC values of ALT (0.59; *P*=0.0004) and CK-18 (0.72; *P*=0.0225; **Figure 4a** and **Table 3**). With regard to the differentiation between borderline NASH and definite NASH, an AUC value of 0.85 was reached by using CatD, and this was significantly higher than the AUC values of ALT (0.57; *P*=0.0011) and CK-18 (0.57; *P*=0.0003; **Figure 4b**). An AUC value of 0.88 for CatD was given for the differentiation between steatosis+borderline NASH vs. NASH, compared with an AUC of 0.52 and 0.53 for ALT and CK-18, respectively (**Figure 4c**). For this differentiation, further statistical analysis revealed that the AUC value of CatD is higher than the AUC values of ALT (*P*<0.0001) and CK-18 (*P*<0.0001; **Table 3**). No significant differences were observed between the AUCs of CatD (AUC: 0.81), ALT (AUC: 0.66), and CK-18 (AUC: 0.74) to distinguish steatosis vs. borderline NASH+NASH (**Figure 4d** and **Table 3**).

The diagnostic performances of CatD, ALT, and CK-18 in the prediction of NASH are depicted in **Supplementary Table S1** online. The best cutoff point for CatD was <18,445 pg/ml, demonstrating the highest sensitivity (100%) and specificity (89.5%). Positive and negative predictive values (PPV and NPV) were calculated and were 92.9% and 100%, respectively. In contrast to CatD, ALT and CK-18 displayed lower sensitivity and specificity rates and less accurate PPV and NPV percentages to predict pediatric NASH.

In short, compared with ALT and CK-18, CatD holds better diagnostic value to predict pediatric NASH and is accurate in making the differentiation between pediatric NASH subjects from those who have steatosis. Similar to steatosis vs. NASH subjects, CatD also improves the prediction of NASH compared with the combination of steatosis+borderline NASH, and also compared with borderline NASH separately.

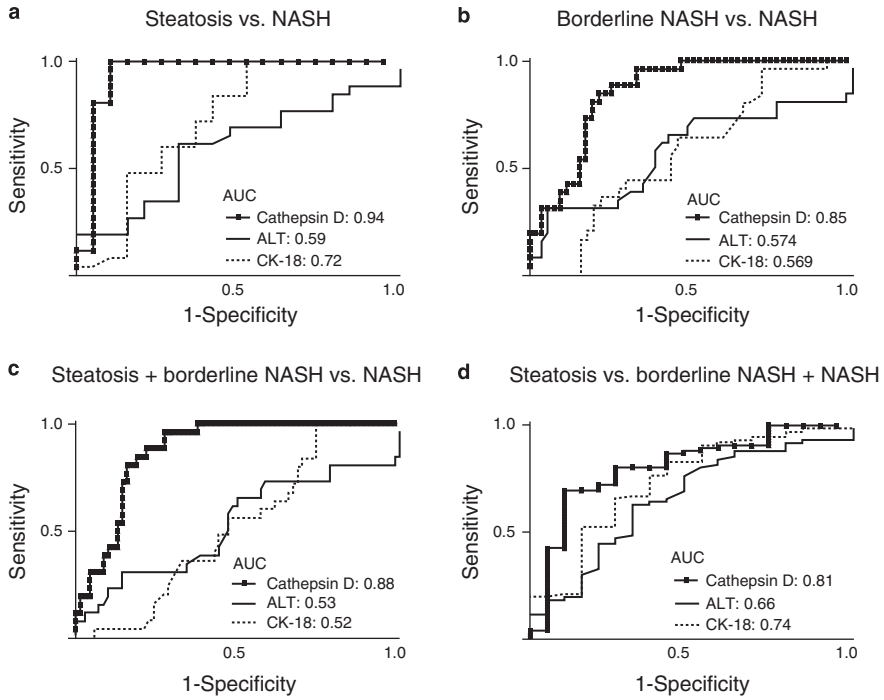

Figure 4. The diagnostic value of cathepsin D, cytokeratin-18 (CK-18), and alanine aminotransferase (ALT) in pediatric nonalcoholic fatty liver disease (NAFLD). Receiver operating characteristic (ROC) curve analysis was performed to assess the area under the curve (AUC) of using plasma cathepsin D, CK-18, or ALT to predict hepatic inflammation for the following comparisons: (**a**) steatosis vs. nonalcoholic steatohepatitis (NASH); (**b**) borderline NASH vs. NASH; (**c**) steatosis combined with borderline NASH vs. NASH; and (**d**) steatosis vs. borderline NASH+NASH.

Table 3. Overview of the calculated *P* values between the different AUCs

	Cathepsin D vs. ALT	Cathepsin D vs. CK-18	CK-18 vs. ALT
Steatosis vs. NASH	***P*=0.0004**	***P*=0.0225**	*P*=0.2840
Borderline NASH vs. NASH	***P*=0.0011**	***P*=0.0003**	*P*=0.9629
Steatosis+borderline NASH vs. NASH	***P*<0.0001**	***P*<0.0001**	*P*=0.9204
Steatosis vs. borderline NASH+NASH	*P*=0.1030	*P*=0.4299	*P*=0.4189

ALT, alanine aminotransferase; AUC, area under curve; CK-18, cytokeratin-18; NASH, nonalcoholic steatohepatitis.
P values in bold indicate statistically significant differences.

Adding CK-18 to CatD improves the diagnostic accuracy to predict pediatric NASH

To explore whether adding a liver-specific marker, CK-18, to CatD would lead to a better prediction of pediatric NASH, we plotted ROC curves of CatD combined with CK-18. Whereas CatD alone demonstrated an AUC of 0.94 for the comparison of steatotic subjects vs. patients with NASH (**Figure 4a**), the combination of CK-18 with CatD resulted in an AUC of 0.998 (**Figure 5a**). Compared with using CatD alone, combining CK-18 with CatD did not increase the AUC values for the differentiations borderline NASH vs. NASH, and steatosis+borderline NASH vs. NASH (**Figures 4b,c** and **5b,c**). When distinguishing steatosis from borderline NASH+NASH patients by using CatD individually, an AUC value of 0.81 was reached (**Figure 4d**). Upon the addition of CK-18, an AUC value of 0.85 was obtained (**Figure 5d**). These data illustrate that adding CK-18 to CatD contributes to reaching an optimal ROC curve for the diagnostic prediction of pediatric steatosis vs. NASH.

DISCUSSION

Diagnosing NASH during childhood is of critical importance to prevent further progression into NAFLD-related cirrhosis and end-stage liver disease during adolescence. Therefore, understanding the mechanisms that cause progression to NASH at an early age is crucial.

With the help of lysosomal enzymes, lysosomes are best known for their primary role in protein degradation. However, lysosomal function is not merely restricted to degradation of proteins, but increasing evidence now demonstrates that the lysosomal compartment also has a role in the immune system and can be seen as secretory vesicles (23,24). Numerous studies have shown that lysosomal cholesterol accumulation inside macrophages is an event that occurs during inflammation and has been detected in NASH as well as in atherosclerosis (7,25). In line, cathepsins have been shown to be significantly involved in mediating the inflammatory response and cholesterol trafficking (13,26,27).

Furthermore, it has been reported that cathepsin expression was impaired in the liver of NAFLD patients, suggesting a pivotal role of

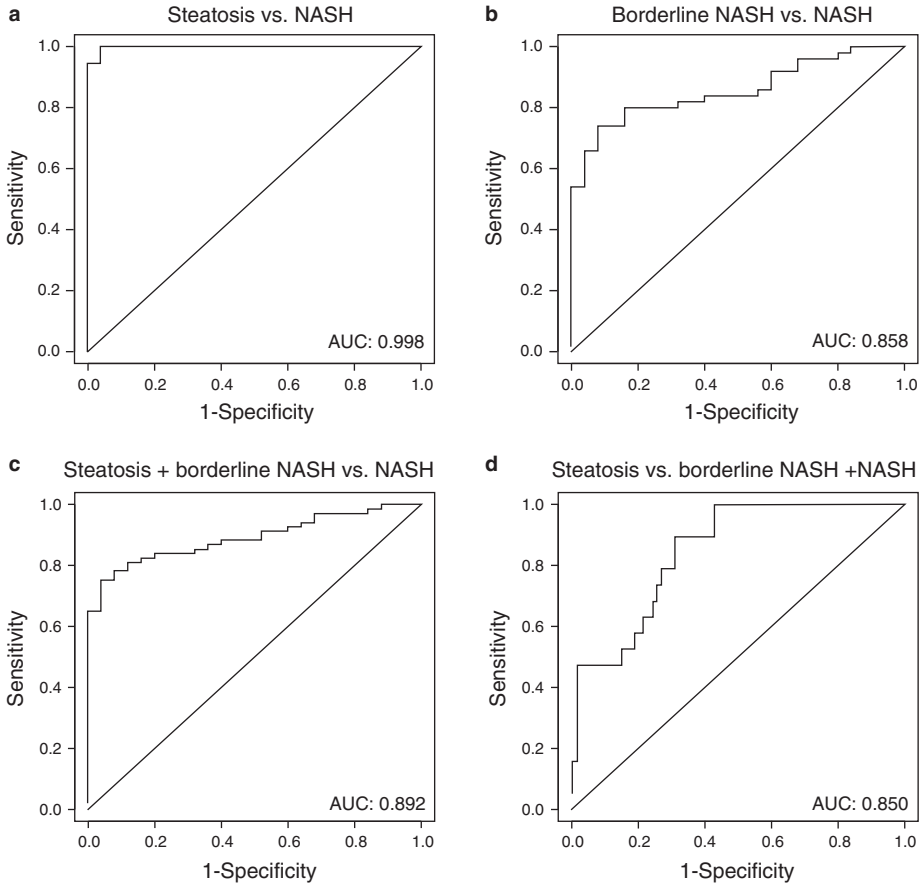

Figure 5. The diagnostic value of adding cytokeratin-18 (CK-18) to cathepsin D. Receiver operating characteristic (ROC) curve analysis was performed to assess the area under the curve (AUC) of using the combination of plasma CK-18 with cathepsin D to predict hepatic inflammation for the following differentiations: (**a**) steatosis vs. nonalcoholic steatohepatitis (NASH); (**b**) borderline NASH vs. NASH; (**c**) steatosis combined with borderline NASH vs. NASH; and (**d**) steatosis vs. borderline NASH+NASH.

these proteins in the setting of liver inflammation (14). In addition, these findings would indicate that cathepsins are more likely to be involved during early stages of NAFLD, rather than a reflection of a late consequence of inflammation. Indeed, in this study, we showed that levels of plasma CatD are decreased at early stages of NASH, whereas these remain similar upon the different stages of fibrosis. This was in contrast to plasma ALT that showed an increase during late-stage fibrosis and was more representative for liver damage. Similarly, a correlation exists between circulating CatD and carotid intima–media thickness, an indicator of atherosclerosis (28). In line with these results, abnormal CatD fractions were associated with a lysosomal storage disease (29), a disease predominantly characterized by chronic systemic inflammation. Thus, it seems that lysosomal cholesterol accumulation in KCs, and its subsequent effect on lysosomal enzyme homeostasis, has a central role in the development of childhood NASH. Although many evidences point to the link between lysosomal enzymes and NASH, the exact mechanisms that lead to the secretion of the lysosomal content in plasma are not yet known.

Lysosomes are able to secrete their content, including lysosomal enzymes, via fusion with the plasma membrane. Such fusion is a calcium-dependent process and has been shown to be sensitive for cholesterol levels. As a result, inducing lysosomal cholesterol accumulation completely inhibited lysosomal exocytosis, suggesting that storage of lysosomal cholesterol has a crucial role in pediatric NASH (30). These data are in line with previous results showing high cholesterol levels in KCs, as was indicated by numerous cholesterol-filled droplets in KCs of livers of NASH patients, whereas livers of steatotic patients did not show this typical foam cell-like phenotype (10). However, foam cell-like structures were so far only detected in NASH livers of adults, and still needs to be confirmed in NASH livers of children.

Excessive cholesterol inside lysosomes can also damage the lysosomal membrane and cause lysosomal membrane permeabilization. In turn, there is leakage of cathepsins into the cytosol, whereby cathepsins can mediate the apoptotic signaling pathway (31). Thus, instead of extracellular secretion, less viable cells remain left to secrete CatD because of increased apoptosis.

The gold standard to diagnose pediatric NASH is still an invasive liver biopsy procedure. However, owing to its invasiveness, biopsies are not performed as a first step. Therefore, clinicians rely on several noninvasive methods to detect NASH in children, such as liver markers (ALT/AST) and imaging techniques (ultrasound,

computed tomography scan and magnetic resonance imaging). Currently, ALT is the primary clinical parameter to evaluate NAFLD in children. However, by using a biopsy-confirmed NAFLD cohort, it was found that AST and ALT have limited predictive power to discriminate between low and high NAS scores in children, with AUC values varying from 0.59 to 0.76 (32). In line, normal ALT levels were observed despite the presence of NAFLD, confirming a low correlation between ALT and NAFLD (33). These data are confirmed in our study by demonstrating low AUC (0.53–0.59) and predictive values (PPV: 72.7% and NPV: 56.5%) for ALT in the discrimination between NASH and no NASH.

A promising serum marker in adults is CK-18 that is representative of hepatocyte apoptosis. Recently, CK-18 was tested in a biopsy-proven children cohort, confirming that CK-18 is also an accurate way of predicting NASH vs. steatosis already during early life (34). Similarly, a second pediatric study confirmed the high diagnostic accuracy for CK-18 in predicting NASH (AUC value: 0.93) and a PPV and NPV of 94% and 72%, respectively (6). Comparable to these studies, we now found a high AUC value (0.72) and similar PPV and NPV values of CK-18 (72% and 100%, respectively) for the detection of NASH vs. steatosis, confirming that CK-18 is accurate at diagnosing NASH in children. However, in our cohort, plasma CatD correlated better with pediatric NAFLD disease progression than CK-18. Altogether, in contrast to other plasma parameters tested for pediatric NASH, our data suggest that plasma CatD is a highly accurate and early noninvasive marker to distinguish steatosis vs. NASH in children. In contrast to CK-18, CatD correlated significantly with NAFLD severity.

Despite our significant findings, the cohort that was used is relatively small in patient size and is of cross-sectional nature. Additional cohorts and future longitudinal studies are essential to validate plasma CatD as a useful tool to detect pediatric hepatic inflammation. Moreover, in view of statistics, a larger sample size would also boost the statistical power. Furthermore, intervention studies should be performed to test whether plasma CatD could be implicated in the clinical follow-up. As obtaining liver biopsies from children with normal aminotransferases is rarely performed, all pediatric individuals in this study demonstrate abnormal aminotransferase levels. As such, children with normal levels of aminotransferases are not included, and, consequently, the level of plasma CatD under healthy conditions is not known. The same restriction holds true for studying CatD in relation to NAFLD in children or adults with a normal body mass index.

Altogether, in this study, we used a well-defined biopsy-proven population of children and evaluated plasma levels of CatD. We have demonstrated for the first time that plasma CatD is significantly decreased in children with NASH compared with children with either steatosis or borderline NASH. Plasma CatD holds high diagnostic value and could be used as a promising noninvasive clinical marker to distinguish pediatric NASH patients from subjects with steatosis, especially upon the addition of CK-18. Furthermore, this is the first human study to show that the lysosomal compartment changes under inflammatory conditions. However, future research is warranted to fully understand the novel role of lysosomes during the development of early NASH. Moreover, whether CatD can be used in clinical practice as a noninvasive marker for NASH should be validated in additional larger well-defined NAFLD cohorts in children and adults.

CONFLICT OF INTEREST

Guarantor of the article: Ronit Shiri-Sverdlov, PhD.

Specific author contributions: S.M.A.W., V.N., and R.S.-S.: study concept and design; S.M.A.W., To.H, Ti.H., V.N., M.L.J.J., P.J.v.G., and R.S.-S.: acquisition of data; S.M.A.W., A.M., P.J.L., V.N., and R.S.-S. (statistical): analysis and interpretation of data; S.M.A.W., V.N., and R.S.-S.: drafting of the manuscript; S.M.A.W., A.C.E.V., M.P.A., W.A.B., A.A., D.L., N.P., V.N., and R.S.-S.: critical revision of the manuscript; M.H.H., G.H.K., A.A., V.N., and R.S.-S.: obtained funding.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ As noninvasive tools are lacking, the current gold standard to diagnose hepatic inflammation in children is an invasive liver biopsy.
- ✓ Previous *in vivo* studies revealed an association between lysosomal cholesterol accumulation in Kupffer cells and hepatic inflammation.

WHAT IS NEW HERE

- ✓ Cathepsin D levels in plasma are significantly reduced in children with biopsy-proven liver inflammation compared to those who have steatosis.
- ✓ Plasma cathepsin D correlated with pediatric nonalcoholic fatty liver disease (NAFLD) progression better than current noninvasive markers.
- ✓ Cathepsin D holds a high predictive value and has the potential to improve noninvasive diagnosis of hepatic inflammation in children.
- ✓ Plasma lysosomal enzymes play an important role during pediatric liver inflammation.

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